ORIGINAL PAPER

Hsp60D is essential for caspase-mediated induced apoptosis in *Drosophila melanogaster*

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Abstract Apart from their roles as chaperones, heat shock proteins are involved in other vital activities including apoptosis with mammalian Hsp60 being ascribed proapoptotic as well as antiapoptotic roles. Using conditional RNAi or overexpression of Hsp60D, a member of the Hsp60 family in *Drosophila melanogaster*, we show that the downregulation of this protein blocks caspase-dependent induced apoptosis. *GMR-Gal4*-driven RNAi for Hsp60D in developing eyes dominantly suppressed cell death caused by expression of Reaper, Hid, or Grim (RHG), the key activators of canonical cell death pathway. Likewise, Hsp60D-RNAi rescued cell death induced by *GMR-Gal4*-directed expression of full-length and activated DRONC. Overexpression of Hsp60D enhanced cell death induced either by directed expression of RHG or DRONC. However, the downregulation of Hsp60D failed to suppress apoptosis caused by unguarded caspases in DIAP1-RNAi flies. Furthermore, in DIAP1-RNAi background, Hsp60D-RNAi also failed to inhibit apoptosis induced by RHG expression. The Hsp60 and DIAP1 show diffuse and distinct granular overlapping distributions in the photoreceptor cells with the bulk of both proteins being outside the mitochondria. Depletion of either of these proteins disrupts the granular distribution of the other. We suggest that in the absence of Hsp60D, DIAP1 is unable to dissociate from effecter and executioner caspases, which thus remain inactive.

Introduction

The Hsp60 family, a major group of the heat shock proteins (Nover 1984), includes stress inducible and constitutively expressed members (McMullin and Hallberg 1988). Hsp60 members are believed to be predominately mitochondrial, although some are also reported in cytosol and in extracellular compartments (Gupta 1990; Retzlaff et al. 1994; Sarkar et al. 2006). As a molecular chaperon, Hsp60 helps in the folding of nascent polypeptides and in the transport of proteins from cytoplasm to organelles (Fink 1999). In addition to typical chaperon function, these proteins are also implicated in other diverse activities like amino acid transport, signal transduction, peptide presentation, regulation of immune system, apoptosis, etc. (Ikawa and Weinberg 1992; Jones et al. 1994; Wells et al. 1997; Woodlock et al. 1997; Sarkar et al. 2006).

Hsp60 family member is generally not induced by heat shock in *Drosophila* tissues (Tissieres et al. 1974) except in Malpighian tubules (Lakhotia and Singh 1989, 1996). The Berkeley *Drosophila* Genome Project has reveled four *Hsp60* genes, named as *Hsp60A*, *Hsp60B*, *Hsp60C*, and *Hsp60D*, respectively (Sarkar and Lakhotia 2005). Studies in our and other laboratories have shown that the Hsp60A, Hsp60B, and Hsp60C proteins have distinct functions in normal development (Kozlova et al. 1997; Perezgasga et al. 1999; Timakov and Zhang 2001; Sarkar and Lakhotia 2005, 2008; Sarkar et al. 2006).

In the present study, we examined the possible functions of the *Hsp60D* (*CG16954*) gene. This gene is located at the 34C1 band, spans a 1.9-kb long region, and codes for a

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putative protein of 448aa, which shows sequence homology with the GroEL of bacteria and human Hsp60 (HSPD1). Transcripts of this gene are ubiquitously found in larval tissues like imaginal discs, salivary glands, etc. (Arya and Lakhotia, unpublished manuscript). In the absence of availability of a mutant allele of this gene, we generated transgenic lines for its conditional RNAi or overexpression. Initial studies showed that ubiquitous ablation or overexpression of Hsp60D using *Act5C-Gal4* or *Tub-Gal4* drivers caused varying degrees of larval or pupal lethality but the surviving flies appeared normal. For tissue-specific expression of the transgenes, we used various Gal4 drivers, including *GMR-Gal4*, which expresses (Ellis et al. 1993; Hay et al. 1994) in developing eye cells posterior to the morphogenetic furrow (MF). It is known that flies homozygous for the *GMR-Gal4* transgene by themselves show some degree of degeneration in eyes (Kramer and Staveley 2003). Unexpectedly, however, the expression of *Hsp60D-RNAi* in the presence of two copies of *GMR-Gal4* transgenes was found to rescue the eye degeneration. Because the eye degeneration in *GMR-Gal4* flies is due to elevated incidence of apoptosis and because Hsp60 proteins have been implicated to have proapoptotic and antiapoptotic roles (Sarkar et al. 2006; Arya et al. 2007), we undertook the present set of experiments to see if Hsp60D protein of *Drosophila melanogaster* has a role in cell death pathway/s.

Programmed cell death by apoptosis is a well-known mechanism through which unwanted cells are removed (Vaux and Korsmeyer 1999). Topmost regulators of the canonical cell death pathway in fly are Reaper (Rpr), Hid, and Grim, collectively often referred to as RHG proteins, which inactivate the inhibitors of caspases and thus trigger apoptosis when expressed (White et al. 1994; Grether et al. 1995; Chen et al. 1996), (Hay and Guo 2006; Arya et al. 2007). The caspases normally remain inactive as unprocessed zymogens and/or bound to inhibitors like *i*nhibitor of *a*poptosis *p*rotein or IAP (Hawkins et al. 1998; Deveraux and Reed 1999; Rodriguez et al. 2002; Salvesen and Duckett 2002). Upon apoptotic stimuli, upstream activators cause the release of inactive caspases from their inhibitors and bring about their proteolytic processing to make active caspases available (Kumar and Colussi 1999; Nicholson 1999). Of the seven caspases known in *Drosophila*, DRONC, DREDD, and STRICA are initiators whereas DRICE, DCP-1, DECAY, and DCP2/DAMM are effectors (Riedl and Shi 2004; Hay and Guo 2006). A number of baculovirus IAP-related proteins have been identified in *Drosophila*, the most extensively studied among them being DIAP1 and DIAP2 (Hay et al. 1995; Duckett et al. 1996; Jones et al. 2000). Ectopic expression of DIAP1 or DIAP2 can suppress cell death induced by RHG proteins or DRONC (Hay et al. 1995; Meier et al. 2000).

In the present paper, we show that the modulation of Hsp60D levels through conditional RNAi or overexpression affects induced apoptosis. Our results suggest that Hsp60D may be necessary for apoptosis through its interaction with DIAP1. A preliminary account of these findings was presented earlier (Arya et al. 2007).

Materials and methods

Generation of *Hsp60D-RNAi* and *UAS-Hsp60DWT* transgenic flies

The base sequence of the *Hsp60D* gene carries short but significant homologies with other *Hsp60* genes of *Drosophila*. Bioinformatic analysis showed that the sequence of a 671-bp *Bgl*II–*Eco*RI (902–1,573bp) fragment from the Hsp60D cDNA clone (AT04835, MRC Gene Services, UK) was unique to Hsp60D. Further bioinformatics analysis was undertaken to check the possibility of "off target effects" (Kulkarni et al. 2006; Perrimon and Mathey-Prevot 2007) of the siRNA fragments produced from the 671-bp dsRNA in the Hsp60D-RNAi transgenic line using two online softwares (http://www.dkfz.de/signaling2/e-rnai/ and http://www.shigen.nig.ac.jp/fly/nigfly/). These analyses confirmed that the 671-bp *Bgl*II–*Eco*RI fragment of the Hsp60D cDNA clone can cause specific RNAi for Hsp60D mRNA without any other mRNA being significantly targeted (data not presented). Therefore, the 671-bp *Bgl*II–*Eco*RI fragment from the Hsp60D cDNA clone (AT04835) was inserted between two oppositely oriented UAS promoters in the *Sym-pUAST* vector (Giordano et al. 2002) to generate the *Hsp60D-RNAi* transgene.

For the overexpression of Hsp60D, the AT04835 Hsp60D cDNA clone was partially digested with *Eco*RI and *Bgl*II to isolate a 2,010-bp fragment, which carries the full coding region of the *Hsp60D* gene. This fragment was cloned in the *pUAST* vector (Brand and Perrimon 1993) to generate *UAS-Hsp60DWT* overexpressing transgene.

To generate transgenic flies, the above two constructs were microinjected separately in *y w*; +; *Δ2–3 Sb ki/Δ2–3 Sb ki* embryos (Cooley et al. 1988). Stable transgenic insertion lines were established by removing the transposase source through appropriate crosses. From among the several independent lines established for the *Hsp60D-RNAi* and the *UAS-Hsp60DWT* transgenes, two lines of each were selected for further studies (see the "Results" section).

Fly stocks and crosses

Fly cultures were maintained at 23 ± 1°C on standard food containing agar, maize powder, yeast, and sugar. Oregon-R$^+$ and Canton-S were used as wild-type strains. Various

transgenic lines such as *GMR-rpr*, *GMR-hid*, *UAS-pro-dronc^w*, *UAS-pro-dronc^s*, *UAS-ΔNdronc*, *GMR-p35* (Meier et al. 2000), *GMR-grim* (Muro et al. 2006), *UAS-DIAP1-RNAi* (Leulier et al. 2006), *UAS-eiger* (Igaki et al. 2002), *GMR-argos* (Freeman 1994), and *Mito-GFP* (Pilling et al. 2006; Goyal et al. 2007) were obtained from various laboratories. *GMR-Gal4* and *Act5C-Gal4* stocks were obtained from the Bloomington stock center. For genetic interaction studies of Hsp60D with various cell death pathway members, the Hsp60D transgenic lines (*UAS-Hsp60DWT* or *UAS-Hsp60D-RNAi*), generated as above, were independently introgressed with the *GMR-Gal4* driver and used for various crosses to generate the desired genotypes. Most of the genetic interactions were carried out with a single copy of the *UAS-Hsp60DWT* or *UAS-Hsp60D-RNAi* transgene unless mentioned otherwise.

Confirmation of transgenic lines

DNA isolation and Southern hybridization

Genomic DNA from 50 flies of each genotype of interest was isolated following Sambrook et al. (1989) and digested with *Bam*H1 restriction enzyme, electrophoretically separated on agarose gel, transferred onto positively charged nylon membrane (Boehringer, Germany), and hybridized with the Hsp60D cDNA (*Eco*RI fragment from the AT04835 clone) probe labeled with digoxigenin by random priming following standard methods. After stringent washes, the blot was processed for colorimetric detection of hybridization as per the manufacturer's instructions (Roche, Germany).

RNA isolation and semiquantitative reverse transcription polymerase chain reaction

Total RNA was isolated from ten adult flies of control (*Act5C-Gal4/CyO*) and transgenic lines expressing two copies of Hsp60D-RNAi or *UAS-Hsp60DWT* under *Act5C-Gal4* driver (*Act5C-Gal4/CyO; Hsp60D-RNAi/Hsp60D-RNAi* and *Act5C-Gal4/CyO; UAS-Hsp60DWT/UAS-Hsp60DWT*, respectively) using Trizol as per the manufacturer's (Life Technology, USA) instructions. The RNA samples were treated with 2U of RNase-free DNAse I (MBI Fermentas, USA) at 37°C for 30min to remove any residual DNA. Semiquantitative reverse transcription polymerase chain reaction (RT-PCR) was performed as per the manufacturer's (Amersham) instructions. Briefly, for cDNA synthesis, 2μg of total RNA, 80pmol of oligo(dT)17 primer, 2U of RNase inhibitor, 500μM of dNTP mix, and 100U of MMLV reverse transcriptase (Amersham) were added to make the final 20μl reaction volume. For PCR amplification, 1/10 volume of the RT product was used for each reaction. *Hsp60D*-specific primers (forward primer: 5′-TGCGATGCTCTAGGTGA GAT-3′ and reverse primer: 5′-ACATCGTAGA TAGGCGGTTC-3′) and glycerol-3-phosphate dehydrogenase (G3PDH) primers (forward primer: 5′-CCACT GCCGAGGAGGTCAACTA-3′ and reverse primer: 5′-GCTCAGGGTGATTGCGTATGCA-3′) were added for PCR. G3PDH is a house-keeping enzyme and has been used in earlier studies (Kanuka et al. 1999) as internal control for RT-PCR. The thermal cycling program included initial denaturation at 94°C for 5min followed by 35 cycles at 94°C for 30s, 60°C for 40s, and 72°C for 1min. Final extension was carried out at 72°C for 5min. The PCR products were run on a 2% agarose gel with 100bp ladder as a molecular weight marker.

RNA–RNA in situ hybridization in third instar larval eye discs

The *AT04835* cDNA clone was digested with *Hin*dIII and *Eco*RI and the 613-bp *Hin*dIII–*Eco*RI fragment (from 960 to 1573bp position of the AT04835 clone), which is unique to the Hsp60D gene (see above), was eluted and ligated with the *Hin*dIII–*Eco*RI-digested *pGEM-3* vector. The resulting subclone, named as p*34C-hsp60D.613*, was linearized by *Eco*RI and used for synthesis of Hsp60D-specific digoxigenin-labeled (Roche, Germany) antisense riboprobe with T7 RNA polymerase. RNA–RNA in situ hybridization (RISH) with intact larval tissues was performed essentially as described earlier (Lakhotia et al. 2001). As a negative control, some of the tissues were treated with RNase (20μg/ml) for 1h at 37°C before hybridization with the Hsp60D riboprobe.

Assay for apoptosis

Acridine orange (AO) staining was used to assay the extent of apoptosis (Spreij 1971; Abrams et al. 1993) in third instar eye imaginal discs from larvae of desired genotype. The discs were dissected in PBS (130mM NaCl, 7mM Na_2HPO_4, 3mM KH_2PO_4, pH7.2) and immediately stained, without any fixation, with 1μg AO/ml of PBS for 3min, following which the discs were washed twice in PBS and mounted in PBS for viewing in LSM510 Meta Zeiss confocal microscope.

Pseudopupil analysis

Adult flies of the desired genotypes were decapitated, and the heads were arranged on a microscope slide. To see the photoreceptor arrangement, corneal neutralization was achieved by adding one drop of immersion oil on the heads (Franceschini 1972). The pseudopupils were viewed in Nikon Ellipse 800 microscope with a ×60 plan-Apo oil

immersion objective (NA 1.4) by illuminating the back of head with a narrow beam of bright light. The images were captured with a Nikon DXM 1200 digital camera.

Nail polish imprints

The nail polish imprints of adult eye surfaces were prepared as described earlier (Arya and Lakhotia 2006). These were examined under DIC optics in a Nikon Ellipse 800 microscope.

Immunostaining and confocal microscopy

For antibody staining, the eye discs were dissected from late third instar larvae of the desired genotypes and fixed in freshly prepared 4% paraformaldehyde in PBS for 20min and processed for immunostaining as described earlier (Prasanth et al. 2000). For detection of the protein/s of interest, the desired primary antibody/antibodies (anti-Hsp60: SPA805 [1:100] and SPA806 [1:50] of Stressgen, Canada; anti-αDIAP1 [1:1,000] from Dr. K. White; anti-GRP75 [1:200] from Dr. S. Ganesh) was/were added singly or in the desired combination to eye discs at the indicated dilution/s. Primary antibody binding was detected with 1:200 dilution of antirabbit or antimouse secondary antibody labeled with Alexa-Fluor 488 (Molecular Probes, USA) or with Cy3 (Sigma), as required. Confocal imaging was carried out on LSM510 Meta Zeiss confocal microscope using appropriate dichroics and filters. All images were assembled using the Adobe Photoshop software.

Results

Hsp60-RNAi and UAS-Hsp60D lines

Among the several transgenic lines generated by us, we selected two each of the *Hsp60D-RNAi* (*Hsp60D-RNAi*[36.d.2] on chromosome 2 and *Hsp60D-RNAi*[41.d.2] on chromosome 3) and *UAS-Hsp60D* (*UAS-Hsp60DWT*[3] on chromosome 2 and *UAS-Hsp60WT*[8.a] on chromosome 3) lines for further studies. To ascertain that the *Hsp60D-RNAi* and *UAS-Hsp60DWT* flies carry the expected transgene, genomic Southern hybridization was carried out. Oregon-R and Canton-S flies were used as controls for identification of the endogenous Hsp60D fragments. Because microinjections of transgenic constructs were performed in Canton-S strain eggs and because the *Hsp60D* gene is located on chromosome 2, the second chromosome transgene stocks carry the chromosome 2 of Canton-S origin, while in the case of third chromosome transgenic lines, the second chromosome was replaced with the chromosome 2 of Oregon-R background during establishment of the transgenic stocks. Therefore,

genomic DNAs from both Oregon-R and Canton-S flies were used as controls. As shown in Fig. 1, *Bam*H1-digested genomic DNAs of Oregon-R and Canton-S flies showed different-sized Hsp60D fragments (8.3 and 6.2kb, respectively), reflecting a polymorphism of genomic organization in the two fly strains. In addition to the endogenous *Hsp60D* fragment corresponding to either Oregon-R or Canton-S controls, the *Hsp60D-RNAi* lines released a single band (3.095kb) whereas the *UAS* lines liberated two (1.96 and 1.18kb) transgene-specific bands due to a *Bam*HI site existing within the Hsp60D cDNA. These results confirmed that the transgenes in these four lines were as expected. To rule out the possibility of insertions other than the desired constructs in these transgenic lines, we performed Southern hybridization using a P-element-specific miniwhite probe. While *Hsp60D-RNAi*[36.d.2], *Hsp60D-RNAi*[41.d.2], and *UAS-Hsp60DWT*[8.a] revealed the presence of only a single P-element insertion, in the case of *UAS-Hsp60DWT*[3], two P-element-specific signals were seen (data not shown). Another Southern blot using an Hsp60D-specific probe confirmed that both the P-element transposons inserted in *UAS-Hsp60DWT*[3] correspond to Hsp60D only (data not shown). These results showed that the transgenic lines carry the expected transgene as a single insertion in the *Hsp60D-RNAi*[36.d.2], *Hsp60D-RNAi*[41.d.2], and *UAS-Hsp60DWT*[8.a] lines but the *UAS-Hsp60DWT*[3] line carried two insertions of the
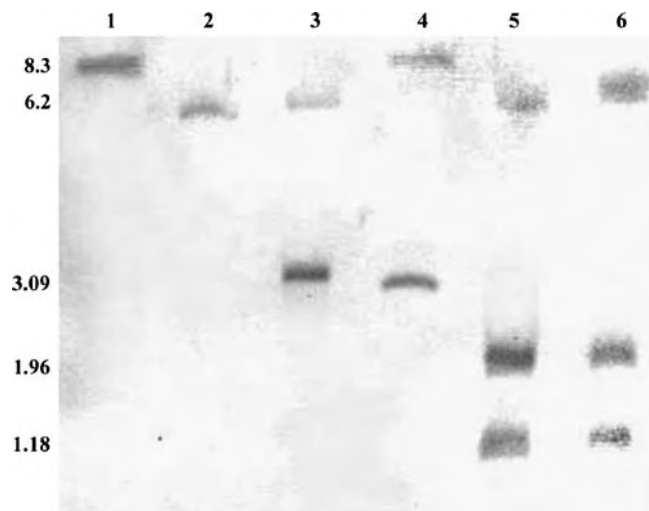

Fig. 1 Genomic DNAs from wild-type Oregon-R (*lane 1*) and Canton-S (*lane 2*), and from *Hsp60D-RNAi*[36.d.2] (*lane 3*), *Hsp60D-RNAi*[41.d.2] (*lane 4*), *UAS-Hsp60DWT*[3] (*lane 5*), *UAS-Hsp60DWT*[8.a] (*lane 6*) homozygous transgenic flies were hybridized with the Hsp60D-specific dig-labeled probe. The 8.3 kb (Oregon-R-specific) or 6.2 kb (Canton-S-specific) bands in different lanes correspond to the endogenous *Hsp60D* gene. The second and third chromosome *Hsp60D-RNAi* homozygotes (*lanes 3* and *4*, respectively) show the expected 3.09 kb transgene-specific fragment while the *UAS-HSp60D* lines with transgene insertion on second or third chromosome (*lanes 5* and *6*, respectively) show the expected two transgene-specific bands (1.96 and 1.18 kb)

transgene and, therefore, was not used further. None of these transgene insertions by themselves, i.e., in absence of any Gal4 driver, showed any phenotype in heterozygous or homozygous conditions. The two Hsp60D-RNAi transgenes (*Hsp60D-RNAi[36.d.2]* and *Hsp60D-RNAi [41.d.2]*) showed comparable effects on phenotypes when driven with any Gal4 driver and, therefore, in the studies reported in this paper, the *Hsp60D-RNAi[41.d.2]* and *UAS-Hsp60DWT[8.a]* lines were used and, in the following discussion, these two lines are referred to as *Hsp60D-RNAi* and *UAS-Hsp60DWT*, respectively.

Levels of *Drosophila* Hsp60D transcripts in different transgenic lines are affected as expected

To confirm that the transgenic lines modulate the levels of the Hsp60D transcripts as expected, they were driven with the ubiquitous *Act5C-Gal4* driver (Ekengren et al. 2001). RT-PCR was performed with RNA isolated from adult flies. As shown in Fig. 2, compared with *Act5C-Gal4/CyO;+/+* (Fig. 2, lane 1) flies, a distinct increase in transcript level was seen in UAS-Hsp60DWT flies (*Act5C-Gal4/CyO; UAS-Hsp60DWT/UAS-Hsp60DWT*; Fig. 2, lane 2). On the other hand, the level of Hsp60D transcripts (long arrow) is detectably reduced following Hsp60D-RNAi expression (*Act5C-Gal4/CyO; Hsp60D-RNAi/Hsp60D-RNAi*; Fig. 2, lane 3). Nearly equal levels of G3PDH amplicons in all the lanes (Fig. 2, short arrow) showed that the alterations in the levels of Hsp60D transcripts were not due to variations in loading of samples.

Hsp60D is essential for normal development

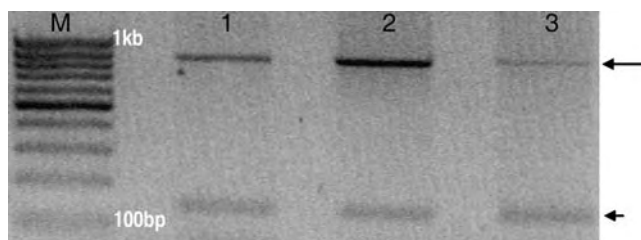To see the effect of global ablation of Hsp60D or its overexpression, the *Hsp60D-RNAi* or the *UAS-Hsp60D*

Fig. 2 Modulation of Hsp60D transcript levels following the expression of the *UAS-Hsp60DWT* or *Hsp60D-RNAi* transgenes with the ubiquitous *Act5C-Gal4* driver. RT-PCR for Hsp60D mRNA produces the 876-bp fragment (*long arrow*) whereas RT-PCR amplicon for the G3PDH mRNA is 140 bp (*small arrow*). *Lane 1* is control (*Act5C-Gal4/CyO; +/+*) showing the normal level of Hsp60D mRNA in adult flies. *Lane 2* is *Act5C-Gal4/CyO; UAS-Hsp60DWT/ UAS-Hsp60DWT* showing increase in Hsp60D mRNA levels and *lane 3* is *Act5C-Gal4/CyO; Hsp60D-RNAi/Hsp60D-RNAi* showing reduction in levels of Hsp60D. Amplicons for G3PDH, used as loading control, show equal loading in all lanes. The *M lane* shows a 100-bp ladder molecular size marker. This is a negative image of an ethidium bromide-stained agarose gel

transgenes were expressed with the *Act5C-Gal4* driver after introgression of either of the transgenes (inserted on chromosome 3) in the *Act5C-Gal4/CyO* stock. Eggs were collected from each of the three stocks, viz., (1) *Act5C-Gal4/CyO; +/+*, (2) *Act5C-Gal4/CyO; Hsp60D-RNAi/ Hsp60D-RNAi*, and (3) *Act5C-Gal4/CyO; UAS-Hsp60D/ UAS-Hsp60D*, and their survival to adult stage counted (Table 1). It may be noted that *Act5C-Gal4/Act5C-Gal4* and *CyO/CyO* progeny die at embryonic or larval stages and thus only 50% of the eggs (*Act5C-Gal4/CyO*) are expected to survive to adult stage. The actual survival of *Act5C-Gal4/CyO* progeny was about 43%, which is significantly less ($P < 0.001$ for χ^2 test) than the expected 50%. It is interesting to note that *Act5C-Gal4*-driven expression of two copies of *Hsp60D-RNAi* transgene further reduced the survival of *Act5C-Gal4/CyO* progeny to 27.6%. On the other hand, overexpression of Hsp60D was somewhat less deleterious (Table 1). The surviving flies in either case had no morphological phenotypes ascribable to ablation or overexpression of the Hsp60D. However, the reduced viability following RNAi for Hsp60D is indicative of an essential role of this gene in normal development.

Hsp60D is expressed in a characteristic pattern in third instar larval eye discs

RISH revealed that besides a low level of ubiquitous presence of Hsp60D transcripts in the cytoplasm of third instar larval eye discs, the transcripts showed a prominent accumulation at the MF and in the differentiating photoreceptor cells posterior to it (Fig. 3a). The specificity of hybridization of the riboprobe with Hsp60D transcripts in eye cells was confirmed by treating the discs with RNase preceding RISH for Hsp60D. These discs did not show any staining (inset in Fig. 3a′). Chromogenic and fluorescent detection of RISH showed that heat shock at 37°C to third instar larval eye discs for 1h did not induce Hsp60D transcripts beyond the control levels (not shown).

Hsp60 family proteins in eye discs form distinct cytoplasmic granules

For immunofluorescence localization of Hsp60 in eye discs, we used two commercially available anti-Hsp60 antibodies, a rabbit polyclonal antibody against Hsp60 of *Heleothis viridis* (SPA805, Stressgen) and a mouse monoclonal antibody against human Hsp60 (SPA806, Stressgen). It is expected that both the antibodies identify all the four Hsp60 forms in *D. melanogaster* (Lakhotia et al. 2002).

The Hsp60 immunostaining pattern generally resembled the Hsp60D transcript distribution pattern in wild-type eye discs (compare Fig. 3a and b with c). Examination of the

Table 1 Global ablation or overexpression of Hsp60D with the *Act5C-Gal4* driver affects survival

Parental genotype	No. of eggs examined	No. (%) of adults xpected to emerge	No. (%) of adults actually emerged
Act5C-Gal4/CyO	939	470 (50)	405 (43.2)
Act5C-Gal4/CyO; Hsp60D-RNAi/Hsp60D-RNAi	1,023	512 (50)	282 (27.6)
Act5C-Gal4/CyO;UAS-Hsp60D/UAS-Hsp60D	1,203	602 (50)	478 (39.7)

immunofluorescently stained discs at higher magnification revealed that in addition to the general diffuse cytoplasmic distribution, both antibodies recognized distinct brightly fluorescing cytoplasmic granules, which were more abundant in the apical regions of the developing photoreceptor units (Fig. 3d,e). To see if the immunofluorescence patterns generated by the two antibodies were identical, we costained wild-type eye discs with both the antibodies. This revealed that while the general cytoplasmic staining was similar (Fig. 3d,e), the granules in photoreceptor cells identified by each antibody were distinct, although mostly adjacent and partially overlapping (Fig. 3f).
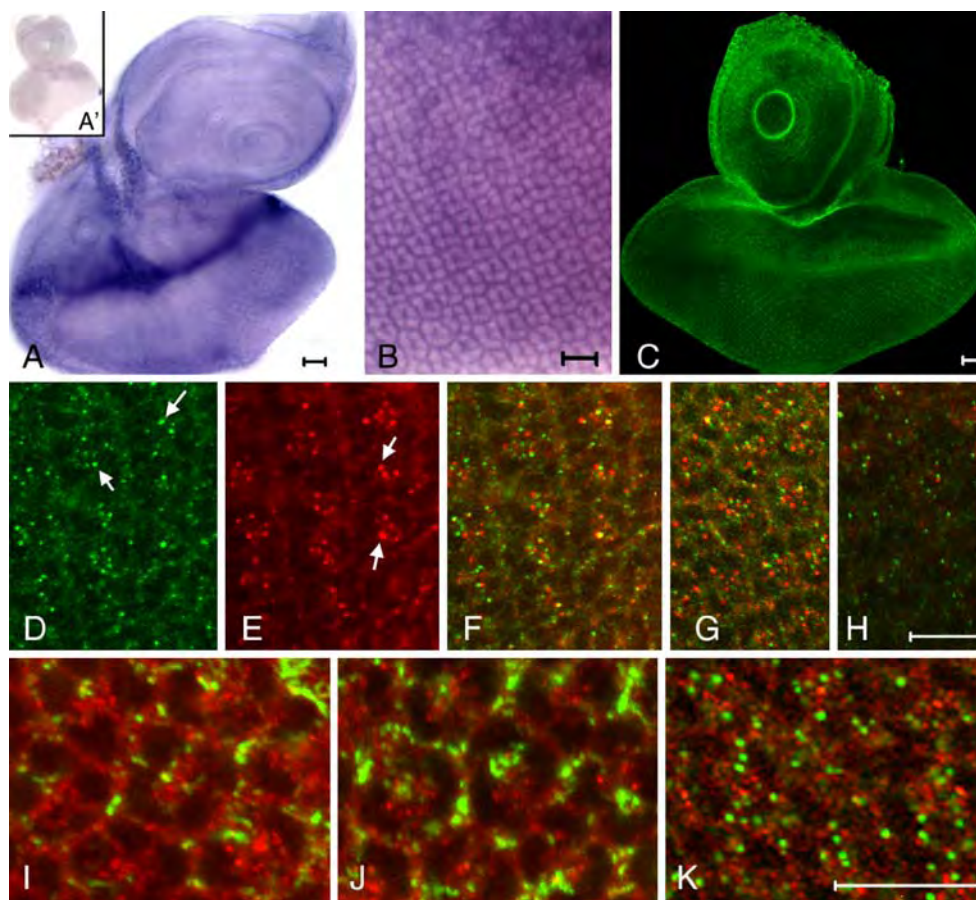
Fig. 3 Localization of Hsp60D transcripts by RISH (**a**, **b**) and Hsp60 protein by immunostaining (**c–k**) in larval eye discs. The Hsp60D transcript levels are high at the MF and the photoreceptor cells posterior to it (**a**). **b** is a higher magnification image of part of the disc in **a** showing patterned distribution of Hsp60D transcripts in photoreceptor units. **a′** (*inset*) shows a disc treated with RNase before RISH. Immunostaining with rabbit anti-Hsp60 (*green*; **c**, **d**, **f**) and mouse anti-Hsp60 (*red*; **e**, **f**) antibodies also shows comparable distribution of Hsp60 proteins in eye discs with distinct Hsp60-positive granules in photoreceptor cells, especially in their apical regions (*arrows*). **f** is a merged image of **d** and **e**. *Hsp60C¹* homozygous eye discs (**g**) show only a slight reduction in Hsp60 proteins recognized by the rabbit and mouse anti-Hsp60 antibodies. On the other hand, the eye discs from *GMR-Gal4; Hsp60DRNAi* larvae (**h**) show greatly reduced diffuse staining with both antibodies while the mouse antibody-recognized (*green*) Hsp60 granules are much less affected than the rabbit antibody-recognized (*red*) ones. Hsp60 granules recognized by either the mouse anti-Hsp60 (*red*; **i**) or the rabbit anti-Hsp60 antibody (*red*; **j**) do not show any significant localization with mitochondria marked by mito-GFP (*green*; **i**, **j**). The mouse anti-Hsp60 (*red*) and rabbit antimitochondrial Grp75 (*green*) also do not colocalize (**k**). Images in **c–k** are single confocal optical sections. Scale bars represent 10 μm (bar in **h** is common for **d–h** and that in **k** is common for **i–k**)

The above finding raised the possibility that the two anti-Hsp60 antibodies may recognize different Hsp60 forms present in *D. melanogaster*. It is known that Hsp60A is expressed ubiquitously but at low levels in most cell types of *Drosophila* (Kozlova et al. 1997) while the Hsp60B is not expressed in any somatic cell (Timakov and Zhang 2001; Srivastava 2004). The *Hsp60C* is expressed ubiquitously, but at low levels, in most cells of larval eye discs (Sarkar and Lakhotia 2005). Therefore, we examined coimmunostaining with both the antibodies in eye discs from *Hsp60C^1* homozygous larvae, which have depleted Hsp60C, and from *GMR-Gal4/GMR-Gal4; Hsp60D-RNAi/Hsp60D-RNAi* larvae, which have depleted Hsp60D (present study). Costaining with the two Hsp60 antibodies showed only a slight reduction in the overall intensity of both the antibodies in *Hsp60C^1* homozygous eye discs although the patterns of diffuse and granular staining were generally similar to those of the wild-type (Fig. 3g). On the other hand, discs expressing the *Hsp60D-RNAi* transgene consistently displayed significantly reduced general diffused staining with both the antibodies. It is interesting to note that while the rabbit anti-Hsp60 antibody reacting Hsp60 granules in their photoreceptor cells were nearly absent, those recognized by the mouse antibody were less affected (Fig. 3h).

The Hsp60 proteins are not restricted to mitochondria in photoreceptor cells

The Hsp60 family proteins are generally believed to be mitochondrial (Ellis and van der Vies 1991; Martin et al. 1995; Bukau and Horwich 1998). Therefore, to check if the Hsp60-positive granules in the cytoplasm of eye disc cells were colocalizing with mitochondria, we used two mitochondrial markers in combination with either of the two Hsp60 antibodies. In one case, larvae expressing GFP in mitochondria (Pilling et al. 2006) were used (Fig. 3i,j), while in the other set, eye discs from wild-type larvae were immunostained with a polyclonal anti-Grp75 antibody (Fig. 3k), which recognizes the Hsp70 family protein localizing in mammalian mitochondrial matrix (Mukamel and Kimchi 2004). As seen in Fig. 3i–k, the Hsp60-rich granules, identified by either of the Hsp60 antibodies, rarely showed any association with mitochondria.

RNAi of Hsp60D rescues degeneration seen in eyes of GMR-Gal4 homozygous adult flies

With a view to understand possible function(s) of the *Hsp60D* gene in *Drosophila* eye development, we used one copy of the *GMR-Gal4* driver to either overexpress or ablate the Hsp60D in developing eyes but did not notice any visible phenotypic consequence following misexpression of this gene. It is interesting to note that, however, we noticed that the eye degeneration (Fig. 4a–c) caused by two copies of *GMR-Gal4* by themselves (Kramer and Staveley 2003) was rescued following ablation of Hsp60D transcripts because the eyes of *GMR-Gal4/GMR-Gal4; Hsp60D-RNAi/Hsp60D-RNAi* flies were indistinguishable from those of the wild-type eyes, externally and in their rhabdomere organization (Fig. 4d–e). On the other hand, overexpression of Hsp60D enhanced the eye degeneration caused by two copies of *GMR-Gal4* resulting in rougher eyes (compare a–b with g–h in Fig. 4). To identify apoptotic cells in eye discs from larvae of different genetic backgrounds, AO staining was carried out as described in the "Materials and methods" section. In agreement with the observed adult eye phenotypes, compared to only *GMR-Gal4* homozygous eye discs, fewer AO-positive cells were seen in the *GMR-Gal4*-driven *Hsp60D-RNAi*-expressing discs (compare c with f in Fig. 4). On the other hand, the AO-positive cells were more frequent in Hsp60D-overexpressing discs (Fig. 4i). These observations raised the possibility that Hsp60D may have some role in cell death pathway.

RNAi of Hsp60D strongly suppresses cell death caused by RHG family proteins

To elucidate the possible role of Hsp60D in apoptosis, the effects of varying levels of Hsp60D on cell death caused by the expression of typical apoptosis-inducing RHG proteins were examined. Unless otherwise mentioned, only single copies of the *Hsp60D-RNAi* or the *UAS-Hsp60DWT* transgenes were driven in the desired genetic backgrounds to see the effects of depletion or overabundance of the Hsp60D protein. As reported earlier (Hay et al. 1994; White et al. 1994; Grether et al. 1995; Chen et al. 1996) and shown in Fig. 5a,d,g,j,m, and q, ectopic expression of any of the RHG proteins under the control of the *GMR*-promoter caused excessive cell death resulting in varyingly malformed and reduced eyes in adult flies. It is interesting to note that coexpression of a single copy of *Hsp60D-RNAi* significantly rescued the eye phenotypes (Fig. 5, compare a, d, g, j, m, q with b, e, h, k, n, r, respectively). *GMR*-driven expression of Hid caused maximum degeneration while that of Grim caused the least (Fig. 5 compare m, q with g, j); correspondingly, the recovery following *Hsp60D-RNAi* coexpression was maximum with Grim and least with Hid (Fig. 5, compare h, k with n, r). Overexpression of *Hsp60D* further enhanced the eye phenotype caused by *GMR-hid* (Fig. 5o,s) resulting in narrower eyes, whereas it had less perceptible effect on Reaper- or Grim-induced eye phenotypes (Fig. 5c,f,i,l).

In agreement with above, a simultaneous overexpression and ablation of Hsp60D transgenes through the *GMR-Gal4* driver in *GMR-hid* background nullified the rescuing effect

Fig. 4 Hsp60D levels modulate eye phenotypes of *GMR-Gal4* homozygous flies. **a**, **d**, and **g** are nail polish imprints while **b**, **e**, and **h** are pseudopupil images of adult eyes; **c**, **f**, and **i** are images of AO-stained third instar larval eye discs. **a**–**c** are from *GMR-Gal4/GMR-Gal4:* +/+, **d**–**f** from *GMR-Gal4/ GMR-Gal4; Hsp60D-RNAi/ Hsp60D-RNAi*, and **g**–**i** from *GMR-Gal4/GMR-Gal4; UAS-Hsp60DWT/UAS-Hsp60DWT* genotypes. Images in **c**, **f**, and **i** are projections of confocal optical sections
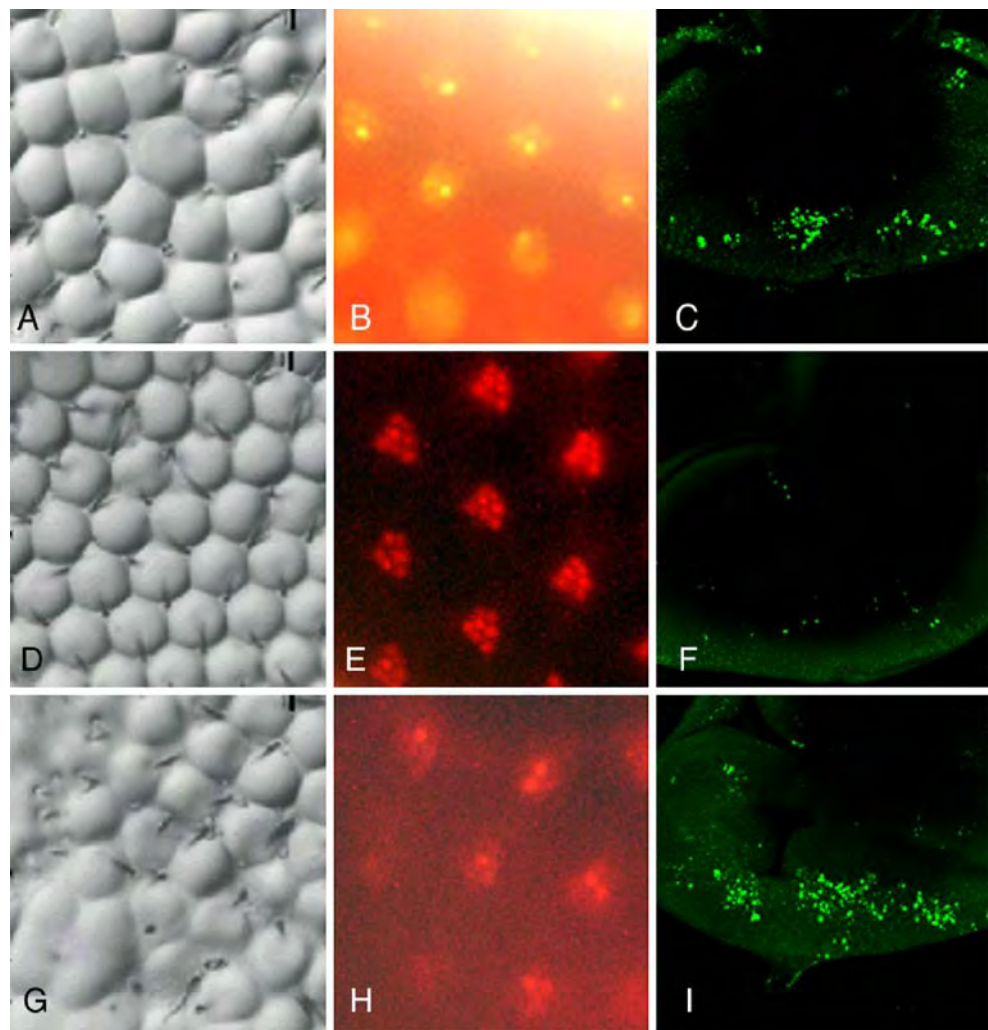
of Hsp60D RNAi because the eyes in *GMR-Gal4/GMR-hid; UAS-Hsp60DWT/UAS-Hsp60D-RNAi* flies showed nearly as much degeneration as in *GMR-hid/+* flies (Fig. 5p,t). This observation confirmed that the modulations of eye phenotypes by *UAS-Hsp60DWT* or *Hsp60D-RNAi* transgenes are indeed due to alterations in the levels of endogenous Hsp60D protein.

Hsp60D-RNAi suppresses apoptosis caused by expression of full-length and processed DRONC

It is known that, under normal conditions, the caspases remain inactive because of binding with DIAP1 and, upon receiving a death signal, the DIAP1 is removed so that the activated caspases bring about cell death. Conditional overexpression of full-length or processed caspases results in increased cell death (Meier et al. 2000), presumably because the inhibitory effects of cellular DIAPs becomes limiting. To check if RNAi of Hsp60D has any effect on cell death caused by overexpression of full-length or

activated caspases, we used transgenic lines expressing full-length *UAS-pro-dronc^w* (weak), *UAS-pro-dronc^s* (strong) or processed *UAS-deltaN-dronc* transgenes. As reported by Meier et al. (2000), eyes of flies ($N = 83$) expressing the *UAS-pro-dronc^w* under the *GMR-Gal4* driver showed near-regular arrays of ommatidia but with an uneven pigmentation (Fig. 6a,e); internally, however, these eyes were degenerate because no pseudopupil image was formed (not shown). *GMR-Gal4*-driven overexpression of strong ($N = 430$) or processed DRONC ($N = 208$) resulted in more severely degenerated and depigmented eyes with reduced head size (Fig. 6c,g and 7a,c, respectively). As already reported (Meier et al. 2000), majority of those expressing *GMR-Gal4*-driven strong (419 out of 430 examined) or processed DRONC (203 out of 208) died as pharates due to their inability to open the pupal case. It is interesting to note that all pupae coexpressing *Hsp60D-RNAi* with *UAS-pro-dronc^s* ($N = 161$) or *UAS-deltaN-dronc* ($N = 97$) eclosed with normal head size. Moreover, coexpression of *Hsp60D-RNAi* with the weak ($N = 127$,

Fig. 5 Hsp60D levels modulate cell death caused by expression of RHG proteins. Eyes of flies of different genotypes as seen by light microscopy (**a–c**, **g–i**, **m–p**) or in nail polish imprints (**d–f**, **j–l**, **q–t**) of *GMR-rpr* (**a**, **d**), *GMR-grim* (**g**, **j**), or *GMR-hid* (**m**, **q**) adults not expressing any of the Hsp60D transgenes (*column 1*) or coexpressing *GMR-Gal4*-driven *Hsp60D-RNAi* (*column 2*) or *UAS-Hsp60DWT* (*column 3*) are shown. Images in the fourth column (**p**, **t**) are from flies expressing *GMR-hid* together with *GMR-Gal4*-driven *Hsp60D-RNAi* and *UAS-Hsp60DWT* transgenes
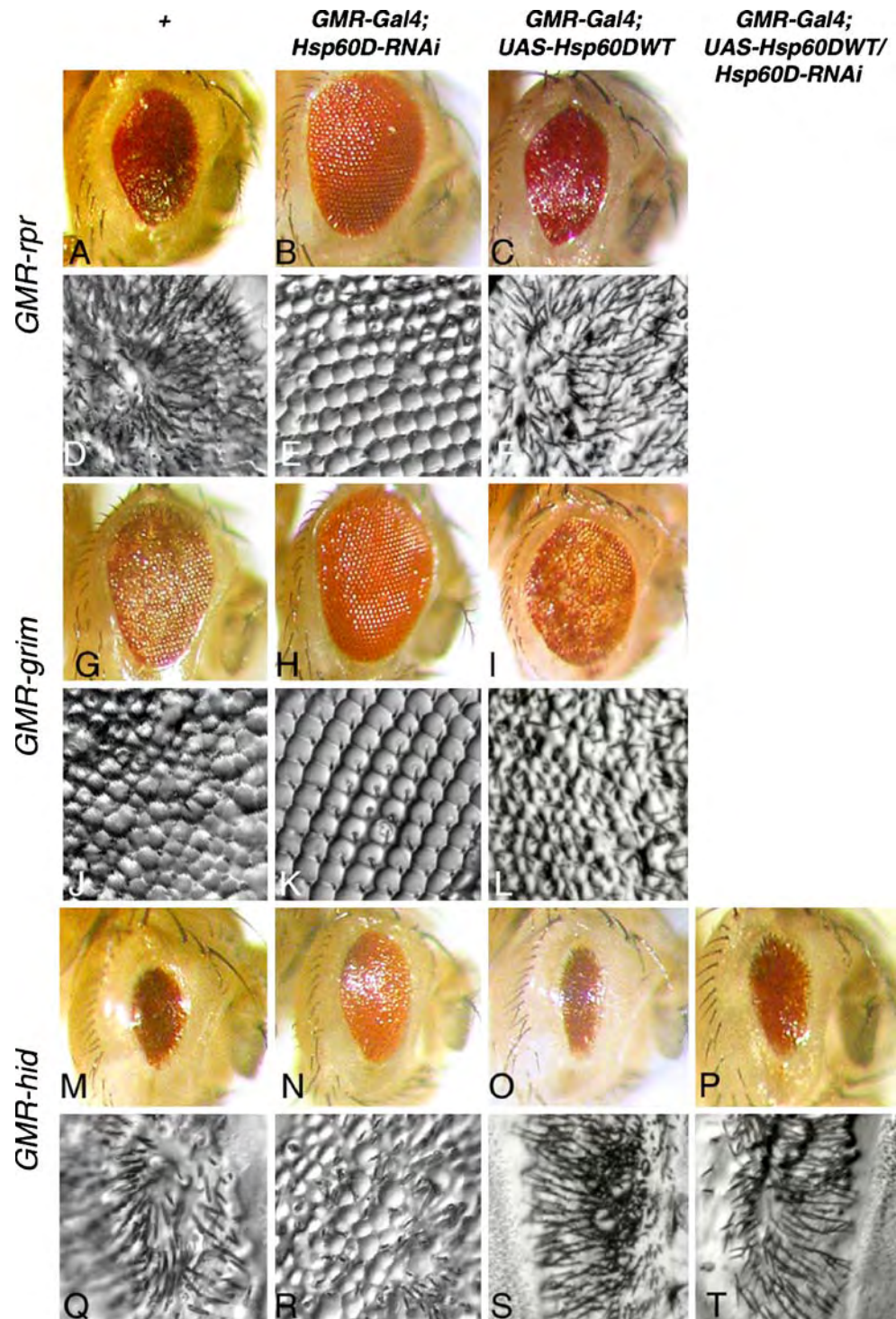
Fig. 6b,f,i) or the strong full-length *DRONC* (Fig. 6d,h,j) or the processed *deltaN-dronc* (Fig. 7b,d) transgene resulted in normal eyes comparable to those of wild-type flies. AO staining of larval eye discs revealed that a large number of dying cells were present in *deltaN-dronc*-expressing third instar eye discs (Fig. 7e), but their number was significantly reduced when the *Hsp60D-RNAi* transgene was coexpressed (Fig. 7h).

Downregulation of Hsp60D is unable to rescue cell death caused by DIAP1-RNAi

Because Hsp60D-RNAi suppressed cell death caused by activated caspases, we further checked if it could also suppress apoptosis following DIAP1-RNAi. Many caspases, including DRONC, become active in the absence of enough DIAP1 and, therefore, cause massive cell death (Leulier et al.
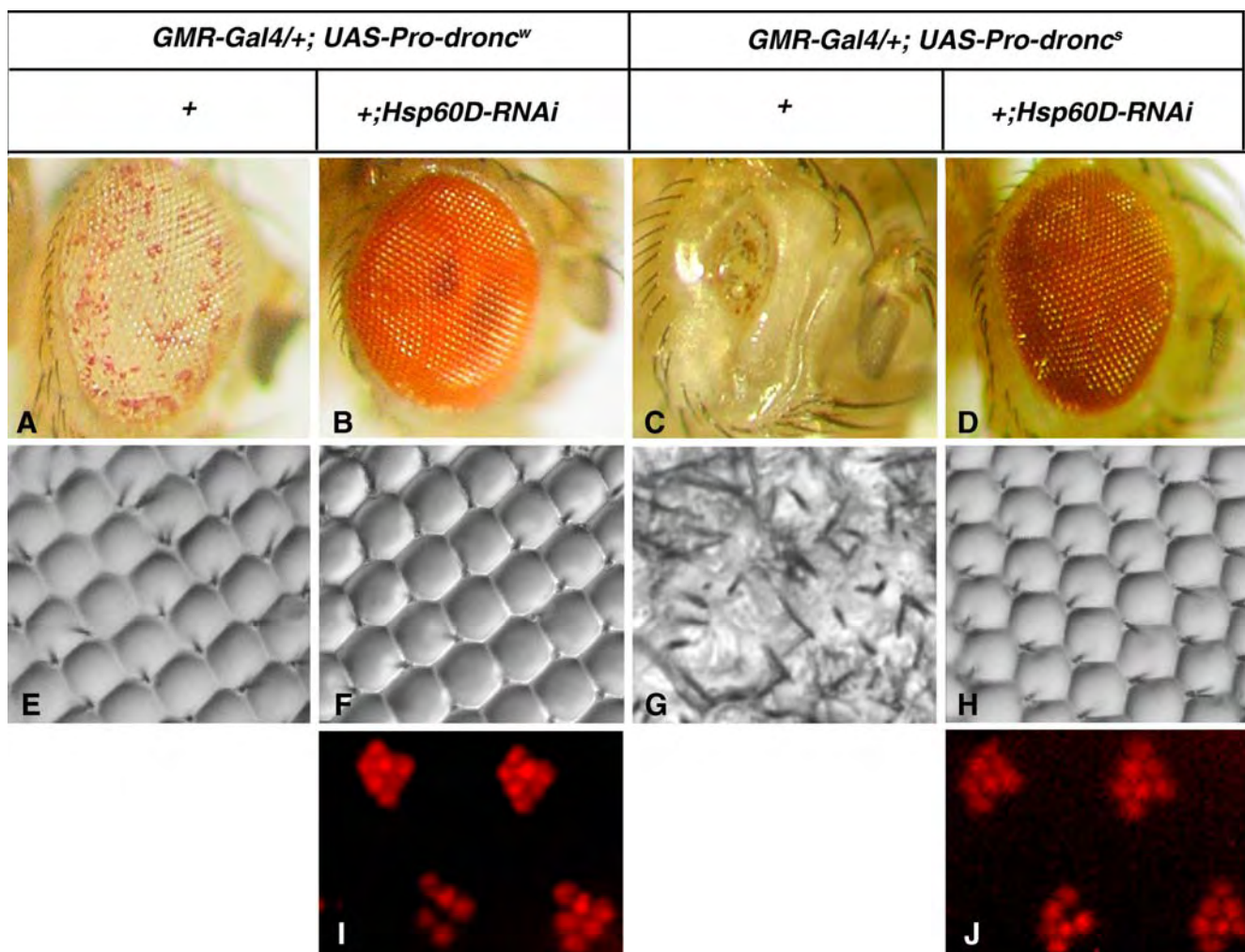
Fig. 6 RNAi of Hsp60D suppresses DRONC-mediated eye degeneration. All transgenes, *UAS-Pro-dronc^w* (**a**, **b**, **e**, **f**, **i**), *UAS-Pro-dronc^s* (**c**, **d**, **g**, **h**, **j**), and/or *Hsp60D-RNAi* (**b**, **d**, **f**, **h**, **i**, **j**), were expressed under control of *GMR-Gal4*. **a–d** are photomicrographs, **e–h** are nail polish imprints, and **i** and **j** are pseudopupil images of adult eyes from flies of different genotypes as indicated on the *top* of each column

2006; Muro et al. 2006). Overexpression of DIAP1 in developing eyes rescues the small eye phenotypes caused by RHG proteins (Hay et al. 1995) or overexpression of DRONC (Meier et al. 2000). In agreement with earlier reports, we observed that *Drosophila* eyes expressing DIAP1-RNAi were reduced, deformed, and partially depigmented (Fig. 8a,d). Intriguingly, unlike in previous cases, Hsp60D-RNAi failed to rescue the reduced and deformed eye phenotype resulting from DIAP1-RNAi (Fig. 8b,e). Even two copies of *Hsp60D-RNAi* transgenes failed to improve the phenotype of *DIAP1-RNAi*-expressing eyes (not shown). Overexpression of Hsp60D mildly enhanced the eye degeneration caused by DIAP1-RNAi (Fig. 8c,f).

Furthermore, ablation of DIAP1 enhanced the eye phenotypes of *GMR-Hid* or *GMR-grim* due to increased apoptosis along with pupal lethality, but this was not suppressed by coexpression of *Hsp60D-RNAi* (not shown). These results strongly suggest that the presence of DIAP1 is

necessary for the inhibitory effect of the Hsp60D-RNAi on apoptosis.

Hsp60 and DIAP1 show adjacent or overlapping distribution in eye disc cell cytoplasm

Coimmunostaining of wild-type eye discs with the mouse anti-Hsp60 antibody and rabbit anti-DIAP1 antibody revealed that like the Hsp60 (see above, Fig. 3d–f), DIAP1 is also present in diffuse manner through the cytoplasm and in greater concentrations in distinct cytoplasmic granules in the apical regions of each developing ommatidial unit (Fig. 9a). It is interesting to note that the Hsp60 granules, recognized by the mouse anti-Hsp60 antibody, and DIAP1 granules were usually adjacent with occasional partial overlap (see Fig. 9b,b′). Because the Hsp60 granules identified by the rabbit anti-Hsp60 antibody also showed a comparable adjacent and sometimes partially overlapping
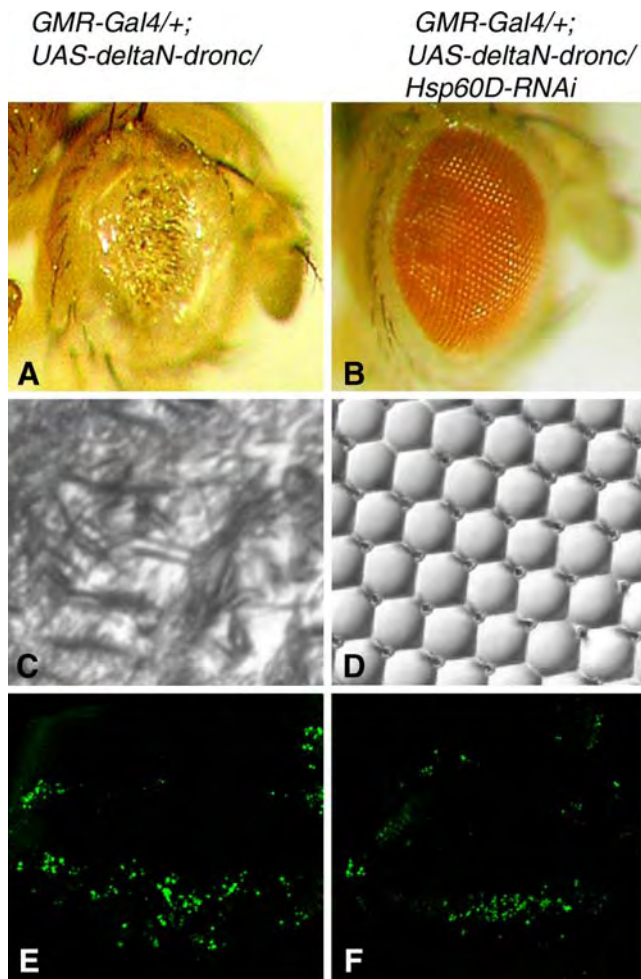
Fig. 7 Hsp60D-RNAi effectively suppresses cell death caused by active DRONC. Photomicrographs (**a**, **b**) or nail polish imprints (**c**, **d**) of eyes of *GMR-Gal4/+; UAS-deltaN-dronc/+* (**a**, **c**) or *GMR-Gal4/+; UAS-deltaN-dronc/Hsp60D-RNAi* (**b**, **d**) flies. **e** and **f** show AO-stained eye discs from *GMR-Gal4/+; UAS-deltaN-dronc/+* (**e**) or *GMR-Gal4/+; UAS-deltaN-dronc/Hsp60D-RNAi* (**f**) third instar larvae

localization with the mouse anti-Hsp60 antibody (Fig. 3f), it is possible that the Hsp60 granules identified by the rabbit antibody may actually show a greater colocalization with the DIAP1 granules. However, because the DIAP1 antibody is also raised in rabbit, it could not be used for conventional coimmunostaining with the rabbit anti-Hsp60 antibody. To circumvent this limitation, to some extent at least, we used an indirect approach to see if the Hsp60 identified by the rabbit anti-Hsp60 antibody actually colocalized or overlapped with DIAP1. Eye discs from wild-type late third instar larvae were first incubated with the rabbit anti-Hsp60 antibody, washed, and incubated with antirabbit Alexa-Fluor 488 conjugated secondary antibody. After washing, the Hsp60-stained discs were divided into two sets. In one set, the discs were directly incubated with antirabbit Cy3 conjugated secondary antibody. In the

second set, the Hsp60-stained eye discs were incubated with the rabbit anti-DIAP1 antibody followed by washing and incubation with antirabbit Cy3 conjugated secondary antibody. The first set of discs would show green fluorescence reflecting Hsp60 recognized by the rabbit anti-Hsp60 antibody while the red fluorescence (excitation at 561nm) in these discs would reflect the extent of Cy3-labeled antirabbit secondary antibody binding with the residual primary rabbit anti-Hsp60 antibody that was not occupied by the antirabbit Alexa-Fluor 488 conjugated secondary antibody. For examining the second set of discs, which were exposed to the two primary rabbit (anti-Hsp60 and anti-DIAP1) and two secondary antirabbit antibodies, the gain of the channel selected for recording red (Cy3) fluorescence was reduced to a level which just eliminated the signal generated by the antirabbit Cy3 conjugated antibody in the first set of discs (Fig. 9d). This reduction in gain was thus expected to remove, in the second set of discs, any signal generated by the antirabbit Cy3 conjugated secondary antibody that binds with the anti-Hsp60 primary antibody while any additional signal in this channel would be expected to represent the binding of this secondary antibody with the anti-DIAP1 primary antibody. The results of this double immunostaining are shown in Fig. 9c–h. It is seen that double staining with rabbit anti-Hsp60 antibody and rabbit anti-DIAP1 antibody resulted in almost complete colocalization of the diffuse and the granular forms of Hsp60 and DIAP1 (Fig. 9h). Granules showing *only* red but no green fluorescence, indicating the absence of Hsp60, were not seen in the second set of discs (Fig. 9h). Comparable results were obtained when the first primary antibody used was the rabbit anti-DIAP1 antibody and the second primary antibody was the rabbit anti-Hsp60 antibody (not shown). These results suggested that all granular forms of DIAP1 in these cells colocalized with those recognized by the rabbit anti-Hsp60 antibody.

To check if the above-noted adjacent/overlapping distribution of these two proteins is of functional significance, we examined the effect of depleting Hsp60D or DIAP1 on their distributions. Coimmunostaining of the eye discs for both the proteins following *GMR-Gal4*-driven expression of *DIAP1-RNAi* or *Hsp60D-RNAi* transgenes showed that the reduction in the level of DIAP1 also affected the Hsp60 protein granules, which were smaller and fewer in such cells (Fig. 9i). Likewise, ablation of Hsp60D by RNAi also resulted in significant reduction of DIAP1 granules (Fig. 9j).

Downregulation of Hsp60D rescues JNK and EGFR mutant eye phenotypes resulting from cell death

Following the above results that RNAi of Hsp60D prevented cell death caused by the direct activation of caspases, we

Fig. 8 Hsp60D-RNAi does not suppress cell death caused by depletion of DIAP1. Photomicrographs (**a**–**c**) and nail polish imprints (**d**–**f**) of eyes of *GMR-Gal4/DIAP1-RNAi* (**a, d**), *GMR-Gal4/DIAP1-RNAi; Hsp60D-RNAi/+* (**b, e**), or *GMR-Gal4/DIAP1-RNAi; UAS-Hsp60DWT/+* (**c, f**) flies
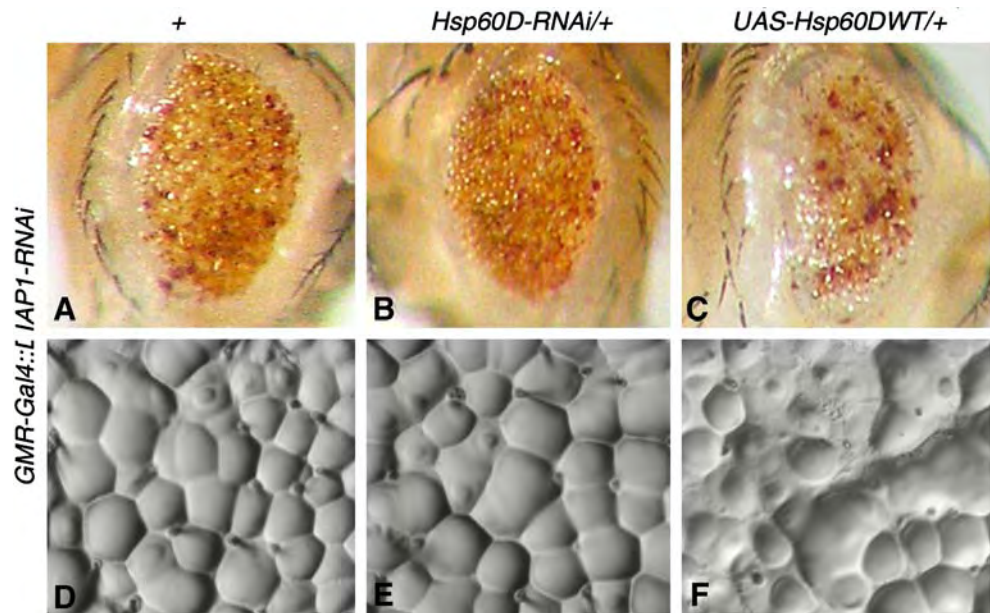
checked if the downregulation of Hsp60D could also suppress the canonical caspase-mediated cell death in JNK or EGFR mutants. Argos is a negative regulator of *Drosophila* EGFR (Schweitzer et al. 1995; Sawamoto et al. 1996). *GMR-Argos* flies showed reduced eyes with irregularly arranged ommatidia (Fig. 10a,c) due to hampered cellular differentiation and activation of cell death (Freeman 1994; Sawamoto et al. 1994; Sawamoto et al. 1998). Coexpression of *Hsp60D-RNAi* suppressed the *GMR-Argos*-induced eye degeneration and resulted in regularly arrayed ommatidial units (Fig. 10b,d).

Eiger is an extracellular activator of Jun-N-terminal kinase (JNK) pathway (Igaki et al. 2002). When expressed ectopically in *Drosophila* eyes, it causes JNK-mediated cell death through the involvement of Hid, Dark, and DRONC (Moreno et al. 2002). As reported by other authors (Igaki et al. 2002; Moreno et al. 2002; Kauppila et al. 2003), ectopic expression of Eiger through *GMR-Gal4* in developing *Drosophila* eye discs resulted in severely damaged eyes (Fig. 10e,g). In this case also, ablation of Hsp60D in Eiger-expressing eye discs resulted in eyes comparable to those of the wild-type (Fig. 10f,h).

Discussion

A general notion about Hsp60 family proteins, as also for the other families of Hsps or heat shock proteins, is that their synthesis is enhanced following cell stress. This is also reflected in the description of the different *Hsp60* genes in *D. melanogaster* genome available at the databases like http://flybase.org. However, as noted earlier, in the case of *Drosophila* larvae, Hsp60 is induced by heat shock only in

the Malpighian tubules (Lakhotia and Singh 1989, 1996). In agreement with this, the present results show that the level of Hsp60D transcripts is not enhanced in heat-shocked eye discs.

Transcripts of *Hsp60A* and *Hsp60C* genes show low abundance in eye disc cells (Kozlova et al. 1997; Sarkar and Lakhotia 2005) while Hsp60B is reported to express only in male germ cells (Timakov and Zhang 2001). However, information available in the databases on the expression of specific genes in different tissues of *D. melanogaster* (e.g., http://flybase.org or http://www.ncbi.nih.gov/geo/ (GDS196)), suggest that the Hsp60A gene transcripts are abundant in larval eye discs while those of the other Hsp60 genes are much less common or absent. On the other hand, our results of RISH clearly show a significant level of Hsp60D transcripts in subsets of differentiating eye discs. A possible reason for this discrepancy may be the significant level of homology in the base sequences of the four Hsp60 genes of *D. melanogaster* (Sarkar and Lakhotia 2005). Therefore, unless gene-specific probes are used, all Hsp60 transcripts may be ascribed to the *Hsp60A* gene because that is presumed to be the ancestral one among the four *Hsp60* genes in the *D. melanogaster* genome (Sarkar and Lakhotia 2005). Thus, notwithstanding the information given in the databases, our results with the Hsp60D gene-specific riboprobe show that this gene's transcripts have high abundance in third instar larval eye discs, especially in the MF and differentiating photoreceptor cells. This is paralleled by the pattern of immunostaining with either of the two anti-Hsp60 antibodies. Together, these suggest that Hsp60D contributes significantly to the pool of Hsp60 family proteins in eye discs. It is intriguing, however, that the two different anti-Hsp60 antibodies (SPA805, polyclonal

Fig. 9 Colocalization of *Drosophila* Hsp60 and DIAP1 in developing photoreceptor cells. Confocal optical sections of wild-type (**a–h**), *GMR-Gal4/ GMR-Gal4; Hsp60D-RNAi/ Hsp60D-RNAi* (**i**), and *GMR-Gal4 DIAP1-RNAi/CyO* (**j**) larval eye discs following immunostaining with rabbit anti-DIAP1 (*red*; **a, b, b'** [*inset*], **g, h–j**), mouse anti-Hsp60 (*green*; **b, b', i,** and **j**) or rabbit anti-Hsp60 (*green*; **c, e, f,** and **h**) primary antibodies. **d** shows the confocal image at 561 nm excitation of a wild-type disc incubated with rabbit anti-Hsp60 primary antibody followed sequentially by binding with antirabbit Alexa-Fluor 488 and antirabbit Cy3 secondary antibodies; the gain in red channel was set at a level which just eliminated any red fluorescence. The same gain setting was used for recording the image in **g** and **h**. Images in **e** and **h** are merges of **c** and **d** and **f** and **g**, respectively. **b, b', i,** and **j** are also merged images for green and red channels. The scale bars, representing 10 μm, are common for each row except the bar in **b'**, which represents 1 μm
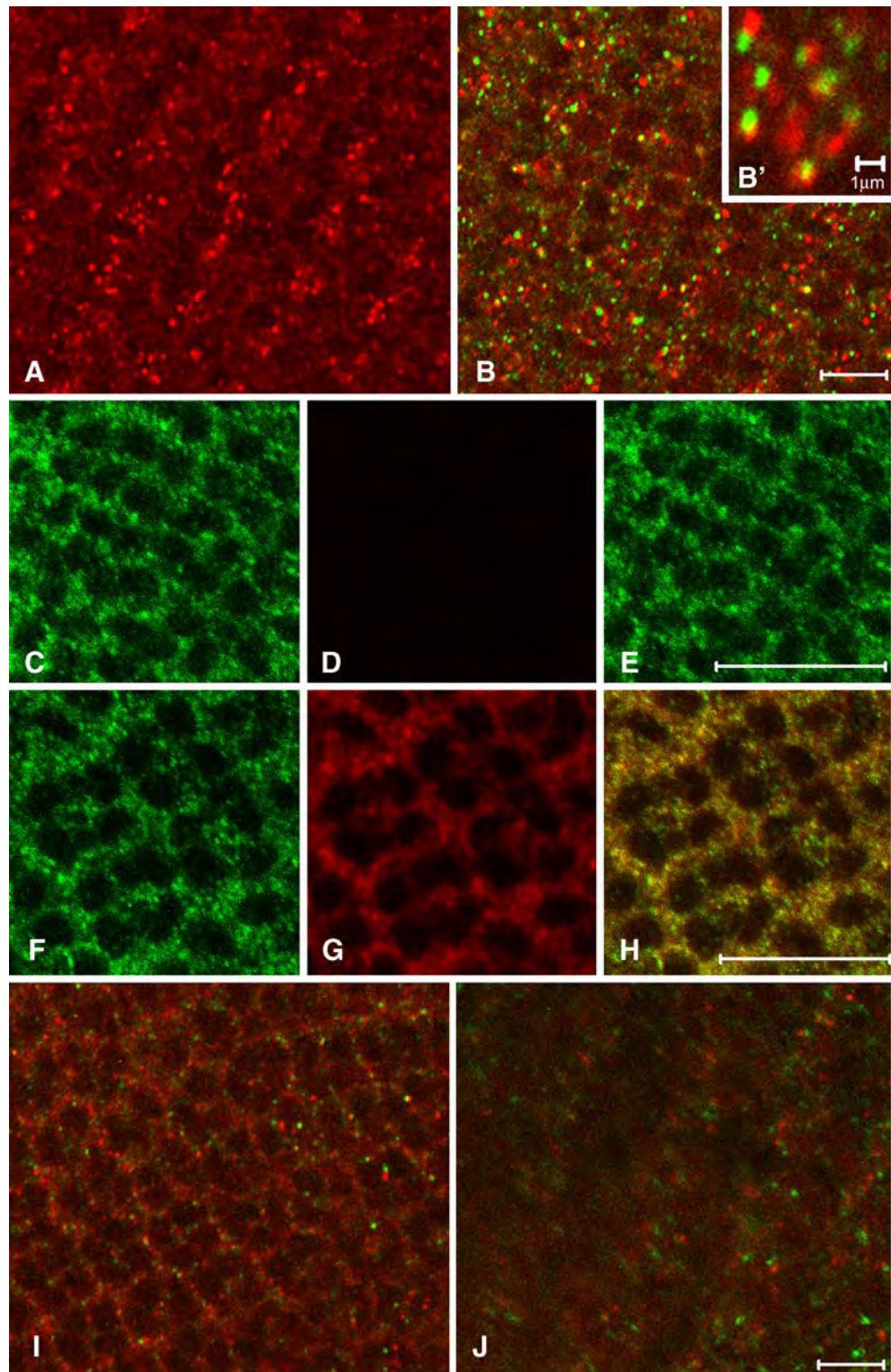
antibody raised in rabbit, and SPA806, a mouse monoclonal antibody; Stressgen), while revealing similar diffuse distribution of Hsp60 in the cytoplasm, decorated different subsets of more brightly fluorescing Hsp60 granules, especially in the apical regions of differentiating photoreceptor cells (Fig. 3d–f). This was unexpected because the two antibodies are believed to detect all the four Hsp60s in *Drosophila* (Lakhotia et al. 2002). Our further studies with these two anti-Hsp60 antibodies in conjunction with conditions that would deplete either Hsp60C (*Hsp60C¹* homozygous condition) or Hsp60D (through RNAi) revealed that the Hsp60 granules identified by the rabbit
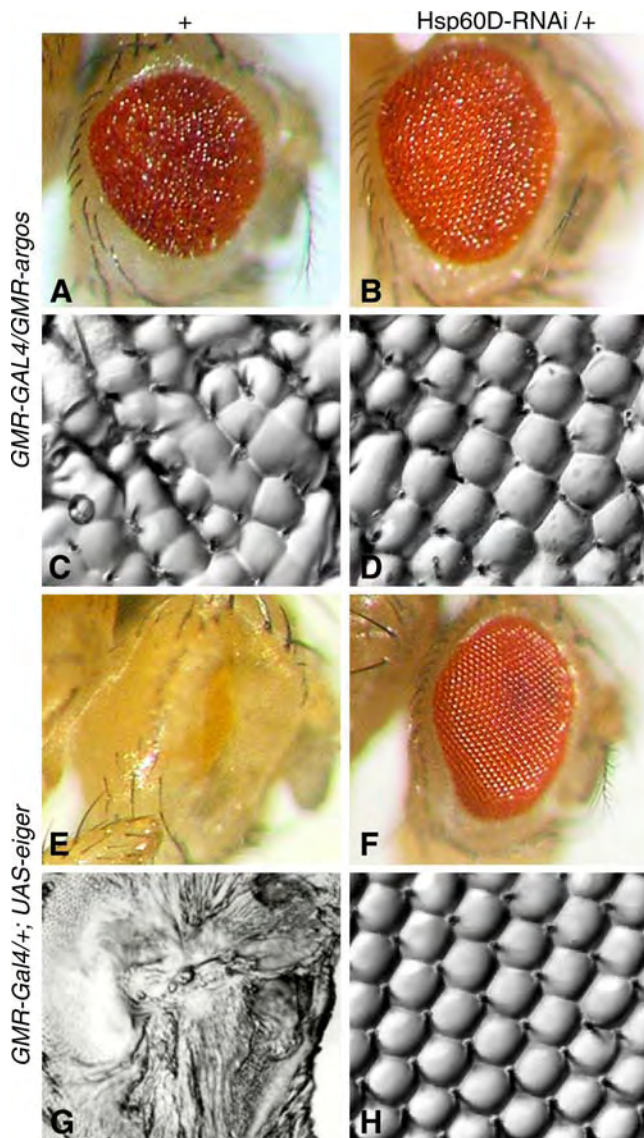
Fig. 10 Hsp60D-RNAi effectively rescues JNK and EGFR mediated cell death. Photomicrographs (a, b, e, f) and nail polish imprints (c, d, g, h) of eyes of *GMR-Gal4/GMR-argos; +/+* (a, c), *GMR-Gal4/GMR-argos; Hsp60D-RNAi/+* (b, d), *GMR-Gal4/+; UAS-Eiger/+* (e, g), and *GMR-Gal4/+; UAS-Eiger/Hsp60D-RNAi* (f, h) flies

anti-Hsp60 antibody were more sensitive to Hsp60D RNAi. Notwithstanding this differential staining of Hsp60 granules by the two antibodies in eye discs and their differential sensitivity of Hsp60D-RNAi, we do not think that the rabbit anti-Hsp60 antibody specifically recognizes the Hsp60D protein because in several other tissues like the trachea, testis, and ovary, the same antibody also recognizes the Hsp60C protein which is depleted by homozygosity of the *Hsp60C[1]* allele (Sarkar and Lakhotia 2005, 2008). It appears more likely that the two antibodies recognize all the four Hsp60 proteins, as expected on their epitope specificities (Lakhotia et al. 2002), but some conformational changes under specific conditions or in specific cells

may differentially affect binding of the two antibodies with one or the other Hsp60 form. This needs further analysis.

Conventionally, the Hsp60 family proteins in animal cells are believed to be mostly mitochondrial, although several studies have shown their presence outside the mitochondria as well (Soltys and Gupta 1999; Pfister et al. 2005; Sarkar et al. 2006; Arya et al. 2007). In the present study, we used two well-known mitochondrial markers but found that none of the Hsp60 granules colocalized or associated with mitochondria, although the more diffusely distributed Hsp60 may overlap with mitochondria. The close proximity of the two types of Hsp60 granules with each other in differentiating photoreceptor cells suggests that they may be part of a larger complex, the identity of which remains to be determined. The apparent colocalization of granular DIAP1 with the rabbit anti-Hsp60 antibody-recognized Hsp60 granules further suggests that DIAP1 is localized in part of this structure. It is possible that conformation of the Hsp60 in the two components of this presumed bipartite structure is different, which makes them differentially recognizable by the two Hsp60 antibodies.

Our present studies reveal a novel role of the Hsp60D protein of *D. melanogaster* in apoptosis. It was seen that apoptosis induced by the expression of Reaper, Grim, or Hid protein by the GMR-promoter in developing eye discs could be dominantly blocked by the depletion of Hsp60D through the coexpression of the *Hsp60D-RNAi* transgene. We have further seen that Hsp60D RNAi dominantly suppressed RHG-induced apoptosis in other cell types as well because *Scabrous-Gal4*-driven expression of Reaper affects the macrochaetae in fly thorax (Igaki et al. 2002) but coexpression of Hsp60D-RNAi rescues the phenotype (data not presented). It is significant that depletion of Hsp60D also dominantly inhibited apoptosis induced either by excess of procaspases or activated caspases or by JNK or EGFR mutants. These observations indicate that Hsp60D is essential for some step/s in the chain of the canonical cell death pathway.

Reaper, Hid, and Grim proteins are believed to induce apoptosis by removing inhibitors of caspases like DIAP1 from the procaspases/caspases (Vernooy et al. 2000). Modes of actions of the three RHG proteins are different in some respects and each of them may also have some distinct roles in apoptosis (Yoo et al. 2002). In this context, it is interesting that the recovery of adult eye structure in Hsp60D-RNAi background was proportional to the initial damage caused by these three proapoptotic factors. This suggests that the presence of Hsp60D protein is essential for the canonical caspase-driven apoptosis but may not be essential for some other paths that lead to apoptosis. Because overexpression of Hsp60D by itself did not result in significantly enhanced apoptosis in developing eye discs, it appears that Hsp60D has a negative role in the

progression of apoptosis such that its presence is essential at one or more steps in apoptosis, but this protein by itself cannot bring about apoptosis.

Hsp60 family members have been suggested to have proapoptotic and prosurvival roles in diverse mammalian cell types (Arya et al. 2007; Chandra et al. 2007). In several cell types, Hsp60 promotes caspase activation and thus has a proapoptotic role (Samali et al. 1999; Xanthoudakis et al. 1999; Chandra et al. 2007) so that the absence of Hsp60 affects maturation of procaspases. In case of prosurvival function, absence of Hsp60 promotes caspase activation (Chandra et al. 2007; Lanneau et al. 2008). Because the downregulation of Hsp60D prevents apoptosis in eye disc cells of *Drosophila* larvae, it may appear that Hsp60D resembles the prodeath role of some mammalian Hsp60 members. However, our results further show that the apparent prodeath role of Hsp60D of *D. melanogaster* differs from that of the mammalian cells. Unlike the mammalian prodeath Hsp60, depletion of Hsp60D blocked apoptosis triggered not only by upstream signals like JNK or EGFR or by excess of procaspase, but also by the expression of activated caspases. In this context, it is significant that apoptosis caused by the absence of functional DIAP1 could not be prevented by the depletion of Hsp60D. DIAP1 associates with procaspases and with activated caspases and thus prevent their downstream activity (Hay and Guo 2006). Keeping in view these observations, we suggest that the removal of DIAP1 from procaspases or activated caspases, following the apoptotic signal, requires the presence of Hsp60D so that when Hsp60D is depleted by RNAi, the procaspases or the activated caspases cannot be released from DIAP1 and thus cannot execute cell death. Our observations on subcellular localization of Hsp60 and DIAP1 indeed suggest a spatially close association of these two proteins. A functional interaction of these two proteins is also indicated by the loss of granular distribution of either of these proteins when any one of these (Hsp60D or DIAP1) is depleted by RNAi.

Lethality associated with global ablation or overexpression of this gene's transcripts through the *Act5C-Gal4* driver does suggest some essential role of this gene in normal development. However, in the absence of any detectable phenotype of adult eyes following its depletion or overexpression through the *GMR-Gal4* driver, except the recovery of damage caused by two copies of the *GMR-Gal4* transgene by Hsp60D-RNAi, this gene's developmental role in eye discs could not be specifically addressed. It is unlikely that this protein serves only as a promoter of apoptosis as and when a cell is triggered to enter the death pathway. Several of the nonapoptotic functions of caspases in *Drosophila* development, like border cell migration during oogenesis, sperm individualization, shaping of aristae, dendrite pruning, development of sensory organ

precursor, etc. (Kuranaga et al. 2006) are mediated via the DIAP1. The DmIKKε degrades DIAP1 which in turn regulates actin dynamics and thus cell morphology, movement, and differentiation of sensory organ precursor cells (Montell 2006; Oshima et al. 2006). The close association of Hsp60 with DIAP1 in granular structures appears to be significant also in *Hsp60D* gene's developmental roles in photoreceptor cells. We have seen (data not presented) that the Hsp60 (and thus DIAP1) granules are present immediately below and abutting the F-actin layer, which is present at the apical regions of photoreceptor cells in late larval eye discs (Arikawa et al. 1990). The close proximity of DIAP1 with F-actin, which plays a critical role in photoreceptor morphogenesis (Benlali et al. 2000; Tepass and Harris 2007), suggests that, as reported in other cell types (Kuranaga et al. 2006; Montell 2006; Oshima et al. 2006), a nonapoptotic function of DIAP1 in eye discs may relate to actin dynamics and thus photoreceptor morphogenesis. Other studies in our laboratory (Sarkar and Lakhotia 2008) have revealed an essential role of Hsp60C in organizing F-actin and other cytoskeletal structures in follicle and germ cells in developing egg chambers. It is, therefore, possible that in differentiating photoreceptor cells, the Hsp60D, together with DIAP1 may regulate the cytoskeletal remodeling of the specific architecture of these highly specialized cells. Absence of an eye phenotype following the targeted depletion or overexpression of Hsp60D in eye disc cells may indicate the existence of alternative paths, e.g., recruitment of other Hsp60 forms and/or critical threshold levels which may not be disrupted following our experimental conditions. The presence of survivors with globally ablated or overexpressed Hsp60D transcripts without any apparent morphological phenotypes also suggests the existence of other mechanisms that can buffer the altered Hsp60D levels.

Further studies are required to understand the specific interaction between Hsp60D and DIAP1 and the mechanism through which one regulates the other in normal development and during induced apoptosis. It will also be interesting to examine if such multiple forms with specialized functions of Hsp60 exist in genomes of other species of *Drosophila* so that their evolutionary significance can be better addressed.

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